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The growth of solid tumors is strictly dependent on new blood vessel formation. This relationship has led to interest in compounds / drugs with potential to block blood vessel growth, or angiogenesis, in tumors. Clinical trials are currently underway to assess the efficacy of anti-angiogenic therapy. To date, these trials have met with variable success; demonstrating promise in some tumor types and not in others. The failure of these agents to affect a broader spectrum of disease is presumed to relate to differences in tissue types. Such results might indicate that alternative drugs might be better suitable to block angiogenesis of specific tumor types. This proposal focuses on the evaluation of thrombospondin-1 (TSP-1) as an anti-angiogenic molecule. In this last year of the proposal, we have found that TSP-1 blocks phosphorylation of MAPK by interfering with the ability of endothelial cells to spread in this substrate. We also found that TSP-1 suppresses focal adhesion kinase phosphorylation and very likely affects endothelial cell migration. Finally, we have continued our screening of functional TSP-1 receptors in endothelial cells derived from both normal and mammary tumors, unlike CD-36, we find that $\alpha v\beta 3$, another reported TSP-1 receptor is expressed at similar levels in both cell types.

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INTRODUCTION

Important note to reviewers:

The present report covers activities of this grant proposal performed during the period of: **December 1st, 1997 to March 30th 1998** (4 months only).

On April 1st, 1998 the principal investigator relocated to UCLA. The project came to a stop until funds were approved for transfer to the new institution. Although the request for transfer was initiated in January 98, the entire process involving two academic institutions and the Department of Defense was only resolved in November 98. In December 98, the grant was finally transferred to UCLA. UCLA has only approved expenditures in January 99.

Therefore the present report is not final. The extension of this proposal was approved until Nov. 99, at this time we will forward a final report.

1. Original Abstract (from grant proposal)

Growth and metastasis of breast cancer is directly dependent on neovascularization. By understanding the mechanisms that control the neovascular response, it may be possible to design therapeutic strategies to selectively prevent or halt pathological growth of vessels and consequently restrain the progression of cancer cells. Despite its general biological significance and pathological relevance, relatively little is known about inhibitors of blood vessel formation. Thrombospondin-1 (TSP1), a glycoprotein originally described as a major component of platelet α-granules, has recently been identified as a negative regulator of angiogenesis. Its relevance for the suppression of vascular growth in tumors has yet to be investigated.

The present proposal was designed to address the role of TSP1 in the neovascularization of mammary tumors. Initially, kinetics of vascular development will be examined in mammary tumors of TSP1-deficient mice and in tumors of control animals. A second set of experiments will focus on the effect of TSP1 in normal and tumor-derived endothelial cells at the cellular and at the molecular level. Specifically, we will investigate the proliferation, invasion, and chemotaxis of normal and tumor-derived endothelial cells in a model of angiogenesis *in vitro*. A final facet of this proposal is directed to investigate the modulation of TSP1 receptors as both populations of cells organize into cords and tubes *in vitro* and to identify the receptor(s) responsible for the generation of signals ultimately responsible for the regulation of endothelial cell behavior in breast cancer.

2. Main goals of this proposal

It has been previously recognized that an increase in the vascular supply plays a central role in tumor progression and metastasis (1-5). In fact in breast cancer, angiogenesis has been acknowledged as a significant indicator of tumor progression that is independent of axillary lymph node status (5-7). Although of recognized relevance, therapeutic approaches have generally excluded treatment of breast cancer by target ablation of neovascular growth; mostly because to date, angiogenic inhibitors have proven

either too generally toxic or not selective to particular vascular beds. This proposal offers a new perspective into the concept of vascular inhibitors by focusing our attention on a natural angiogenic inhibitor present in normal mammary glands: the glycoprotein thrombospondin-1 (TSP1). According to our preliminary data, TSP1 seems to be suppressed during pathological neovascularization of breast tumors, therefore it is our premise that an exogenous supply of TSP1 should be effective and non-toxic. In addition, TSP1 seems to be specific to steroid-dependent tissues, which could potentially offer selectivity in the inhibition of mammary vessels.

This proposal offers a potentially exciting avenue for the identification of a natural inhibitor of capillary growth for the treatment of human breast cancer. The successful completion of this research project will:

1. determine whether the lack of TSP1 facilitates tumor progression and enhancement of vascular growth;
2. identify cellular mechanism(s) by which TSP1 inhibits angiogenesis in endothelial cells;
3. identify the receptor(s) involved in the modulation of endothelial cell behavior and examine the intracellular signaling mechanisms;
4. determine whether TSP1 could be a selective marker for tumor-associated angiogenesis, and more importantly,
5. determine whether the regulation of TSP1 gene can provide a natural pathway for the clinical treatment of breast cancer.

BODY

EXPERIMENTAL METHODS / AIMS (as presented in original proposal)

A. Is the lack of TSP1 associated with growth and metastasis of malignant tumors?

Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice.

Experimental Design/Methodology:

1. Generate mammary tumors in TSP1-deficient (tsp/tsp$^-$) mice by mating of TSP1 knock-out homozygotes with mice carrying the MMTV c-*neu* transgene

2. Analysis of the vascular bed, as well as rate of capillary extension/mm^2 of neoplastic tissue will be obtained by: a) confocal laser analysis coupled with three-dimensional reconstruction, b) determination of hemoglobin and c) endothelial cell markers. Values obtained from TSP1-deficient and from control *neu* animals will be compared.

Overall growth of the tumors and rate of metastasis will also be directly assessed and correlated with control values. Data from these experiments will concurrently provide important information on the relationship between capillary density and tumor expansion.

Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice.

Experimental Design/Methodology:

1. Slow-release pellets of TSP1 protein will be implanted in the mammary fat pads of TSP1-deficient mice carrying the MMTVc-*neu* transgene.

2. Vascular progression in tumors will be determined and compared to control-*neu* mice.

3. In addition, the localization of exogenous TSP1 protein and its half life in tumors will be assessed to gain information on the fate of exogenous TSP1 in mammary tumors.

B. What are the specific effects of TSP1 on endothelial cells engaged in angiogenesis?

Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response.

1. Endothelial cells (EC) from normal mouse mammary gland and from mammary tumors will be isolated and characterized for their proliferation rate, secretory profile, and angiogenic potential.

2. Exogenous TSP1 will be added to EC at confluence or to cells undergoing angiogenesis *in vitro*. These experiments will be performed in both tumor-derived as well as control cells. We will determine the effect of this addition on: a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1.

1. The presence of TSP receptors will be assessed in cultures of confluent EC, as well as in angiogenic cultures, by direct binding assays.
2. Modulation of receptor number will be analyzed after addition of TSP1.
3. Neutralizing experiments with specific anti-TSP receptor antibodies will be performed to determine which of the five recognized receptors mediates an anti-angiogenic response.

RESULTS

STATEMENT OF WORK (as presented in original proposal)

Task 1, Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice, Months 1-24

a. Generate TSP1-deficient mice containing mammary tumors
b. Tumors from experimental and from control animals will be harvested and measured
c. Analyze the frame-work of capillaries in both experimental settings by morphometry
d. Analyze the frame-work of capillaries in both experimental settings using a biochemical strategy.

Task 2, Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice, Months 12-30

a. Preliminary experiments:

Determine the rate of release of [^{125}I]-TSP1 and determine its half life in mammary tumors. Adjust experimental conditions to accommodate these results.

b. Implant capsules containing TSP1 into the mammary tumors of TSP1-deficient mice

c. Determine the effect of TSP1 on the vascular density of mammary tumors as performed in Task #1 (c and d).

Task 3, Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response, Months 23-40.

a. Isolate and characterize endothelial cells from normal and from tumor-containing mammary glands for their proliferation rate, secretory profile, and angiogenic potential

b. Add TSP1 to endothelial cells from normal and tumor of the mammary gland and assess: a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

Task 4, Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1, Months 30-48.

a. Characterize and compare the spectrum of TSP1 receptors expressed by normal and tumor-derived endothelial cells at confluence and after they organize into cords and tubes.

b. Perform Western blots to determine whether one or more of the previously characterized receptors is modulated

The presence of TSP receptors will be assessed in cultures of confluent EC, as well as in angiogenic cultures, by direct binding assays. Modulation of receptor number will be analyzed after addition of TSP1. Neutralizing experiments with specific antibodies will be performed to determine which of the five receptors identified for TSP1 mediates an anti-angiogenic response.

SUMMARY OF ACCOMPLISHMENTS -

Nov. 1994 - Dec. 1997

Task 1-	Months 1-24	Completed
Task 2-	Months 12-30	Completed
Task 3-	Months 23-40	In progress
Task 4.	Months 30-48	In progress

Dec. 1997 – March 1998

Task 3 – We performed experiments to determine the effects of TSP1 on attachment, migration / chemotaxis and expanded the scope of these experiments to further understand the mechanism by which TSP1 affects these endothelial functions (see below).

Completion of this task is only dependent upon experiments to assess the effect of TSP1 on the secretion of other extracellular matrix proteins by endothelial cells.

Task 4 – We have established reproducible conditions to generate cord-like structures on endothelial cells isolated from both normal and tumor tissues. Also, we continued the evaluation of the level of TSP1 receptors in normal and tumor endothelial cells. In contrast to CD36, which as communicated in the previous report was found downregulated in tumor cells, we found that $\alpha v \beta 3$, another receptor for TSP1 was not altered significantly in either endothelial cell type.

<u>**TASKS IN PROGRESS - POINT-TO POINT DISCUSSION**</u>

Task 3, Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response, Months 23-40.

a. <u>Isolate and characterize endothelial cells from normal and from tumor-containing mammary glands for their proliferation rate, secretory profile, and angiogenic potential</u>

Accomplished in year 3.

b. <u>Add TSP1 to endothelial cells from normal and tumor of the mammary gland and assess a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.</u>

Assessment of proliferation was accomplished in Year 3.

During the first quarter of the fourth year we concluded the experiments related to migration and chemotaxis. As predicted, TSP1 inhibited attachment and migration of endothelial cells from normal mammary gland. Similar results were also found with endothelial cells isolated from mammary tumors. In addition, we found that endothelial cells did not spread on TSP1 (Figure 1A). Failure to spread appears to be directly correlated with cell cycle progression, in fact, cells plated on TSP1 do not phosphorylate MAPK (Figure 1B) and are unable to undergo G1 – S transition.

We decided to pursue further these observations and evaluated the effects of TSP1 on the phosphorylation of focal adhesion kinase (FAK) and paxillin, both proteins involved in the interaction of integrins with the cytoskeleton. We found that TSP1 suppressed the phosphorylation of both FAK and paxillin in comparison to cells plated on either collagen or fibronectin (Figure 2). These data supports the effect of TSP1 as an inhibitor of angiogenesis. Cell-extracellular matrix interactions are relevant for migration and proliferation. It is likely that TSP1 suppresses both these interactions by interfering with the signaling cascade initiated upon spreading of endothelial cells on extracellular matrix components.

Task 4, Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1- Months 30-48.

a. Characterize and compare the spectrum of TSP1 receptors expressed by normal and tumor-derived endothelial cells at confluence and after they organize into cords and tubes.

This task is still in progress, while in Boston, we set up the culture conditions that enable the organization of cord-like structures from endothelial cells based on our previous experience on this system (8-10). We were successfully able to promote the organization of tube-like structures in both normal and tumor-derived endothelial cells. We have now, at UCLA, re-established these culture conditions and will initiate isolation of protein to perform Western analysis with antibodies against TSP1 receptors.

b. Perform Western blots to determine whether one or more of the previously characterized receptors is modulated

In year 3 we had performed Westerns blots that assessed the levels of CD-36 in both normal and tumor derived endothelial cells (at subconfluency). Interestingly, we found significant decrease in the levels of CD-36 in tumor-derived endothelial cells. This could likely be an explanation for the increased proliferation and angiogenic response of endothelial cells in tumors and establishes a clear and important difference between endothelial cells in normal and tumor tissues.

During the first four months of year 4, we have continued this evaluation and determined the levels of $\alpha v\beta 3$, another reported receptor for TSP1. In contrast to CD-36, we do not find that $\alpha v\beta 3$ levels are altered (Figure 3). Data on CD36 has been presented in the previous report.

We have now (as of Jan 4th, 1999) re-initiated experiments related to this grant proposal and have 11 months (January to November) to conclude tasks 3 and 4. We are confident that we will meet this deadline and we should also be able to write the two manuscripts that will result from the completion of these tasks.

CONCLUSIONS

From the activities performed during the first quarter of year 4, these are our conclusions:

1. The mechanism by which TSP1 suppresses angiogenesis is likely to include a combination of effects related to inhibition of endothelial cell proliferation and attachment.
2. The receptor involved in these effects is likely to be CD-36 and not $\alpha v\beta 3$, although examination of additional TSP1 receptors have not been concluded at this point.

PRESENTATIONS AND PUBLICATIONS OF FINDINGS RELATED TO THIS GRANT PROPOSAL (year 1997)

A. Presentations

The PI was an invited speaker and presented work related to this proposal in the following meetings:

1) Gordon conference on Vascular Biology, New Hampshire, June 1998.

2) Anti-Cancer Proteins and Drugs: Structure, Function and Design. New York Academy of Sciences Meeting. New York, NY, November 1998.

B. Publications Related to this project

1. **Iruela-Arispe, M.L.**, Vazquez, F., and Ortega, M.A. 1999. Anti-angiogenic domains shared by thrombospondins and ADAM/TS proteins. In: Anti-Cancer Proteins & Drugs: Structure, Function & Design. The New York Academy of Sciences.

2. **Iruela-Arispe, M.L.,** Ortega, M.A., and Vazquez, F. 1999. Anti-angiogenic domain of thrombospondin. In: Angiogenesis in Health and Disease: Basic Mechanisms and Clinical Applications. Gabor Rubany, Editor.

3. **Iruela-Arispe, M.L.,** Lombardo, M., Krutzsch, H.C., Lawler, J., and Roberts, D.D. 1999. Inhibition of angiogenesis by thrombospondin-1 is mediated by two independent regions within the type 1 repeats. Circulation, *in press.*

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5. Ortega, M.A. and **Iruela-Arispe, M.L.** 1999. Thrombospondin-1 is an endogenous regulator of angiogenesis in the mammary gland. J. Cell Biol. Submitted.

FIGURE LEGENDS

Figure 1. Thrombospondin modulates the spreading of endothelial cells. A. Endothelial cells from mammary glands were plated on: (1) Fibronectin, (2) Type I collagen; or (3) thrombospondin and allowed to spread for 30min. Phase contrast images showed degree of spreading in all substrates. Note that cells do not spread on thrombospondin and remain round. B. Mammary gland endothelial cells were plated on either fibronectin or thrombospondin. At the indicated time points, cells were harvested in laemmli buffer, subjected to SDS-PAGE, and evaluated for the state of MAPK phosphorylation by Western blot. The arrows indicate both p42 and p44 phsophorylated forms of MAPK.

Figure 2. Thrombospondin suppresses FAK phosphorylation on endothelial cells. Mammary gland derived endothelial cells were plated on specific extracellular matrix proteins allowed to attach for 1h and harvested in RIPA buffer. Cell extracts were immunoprecipitated with anti-FAK andtibodies and evaluated for the state of FAK phosphorylation. The lanes differ on the substrate used to plate cells and are: (1) fibronectin; (2) thrombospondin; (3) type I collagen; (4) tenascin; (5) laminin; (6) SPARC; and (7) type III collagen. The resulting western blot was evaluated with an anti-phosphotyrosine antibody (PPY)and an anti-FAK antibody to assess levels of FAK post-immunoprecipitation.

Figure 3. Expression of αvß3 on endothelial cells from tumors and from normal mammary glands.
Endothelial cells were isolated from normal (1-3) or from tumor-bearing (4-6) mammary glands. Equal number of cells were subjected to SDS-PAGE and the resulting Western blot was probed with an antibody to both αv and ß3. The arrows indicates the migration and positive immunological reaction.

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List of Personnel involved in this project
For year 3 - 10/96 to 10/97

Dr. Luisa Iruela-Arispe	- Principal Investigator - 20%
Dr. Mariasun Ortega	- Post-doctoral fellow - 100%
Ms. Sarah Oikemus	- Research Assitant - 40%

FIGURE 1

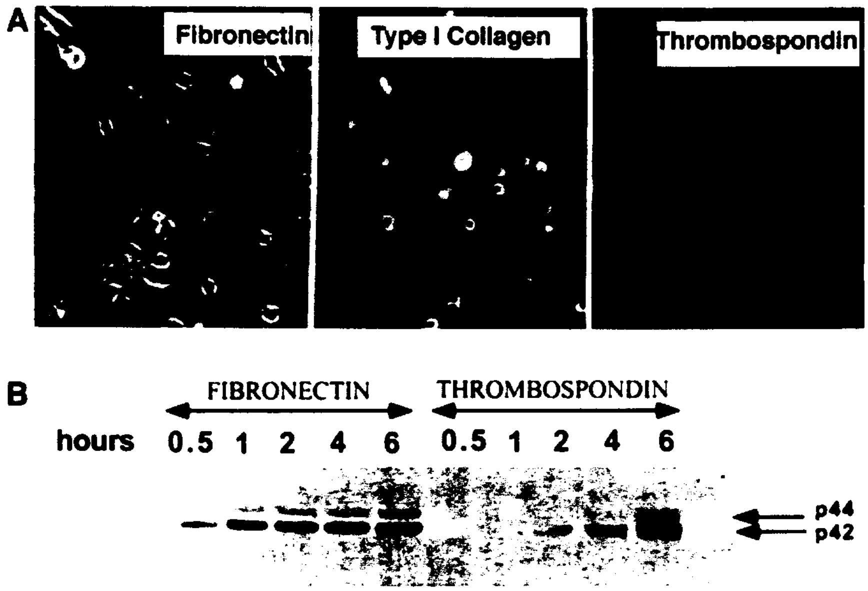

FIGURE 2

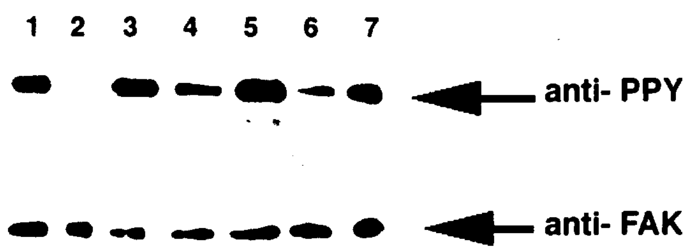

FIGURE 3